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मानक

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IS 5887-3 (1999): Methods for Detection of Bacteria Responsible for Food Poisoning, Part 3: General Guidance on Methods for the Detection of Salmonella [FAD 15: Food Hygiene, Safety Management and Other Systems]



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Bhartrhari—Nitiśatakam

“Knowledge is such a treasure which cannot be stolen”



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भारतीय मानक

खाद्य विषाक्तता उत्पन्न करने वाले जीवाणुओं के  
संसूचन की पद्धतियाँ

भाग 3 सालमोनेलिया के संसूचन के लिए पद्धति पर सामान्य मार्गदर्शी सिद्धान्त  
( दूसरा पुनरीक्षण )

*Indian Standard*

METHODS FOR DETECTION OF BACTERIA  
RESPONSIBLE FOR FOOD POISONING

PART 3 GENERAL GUIDANCE ON METHODS FOR THE DETECTION OF *SALMONELLA*  
( *Second Revision* )

ICS 07.100.30

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NEW DELHI 110002

## NATIONAL FOREWORD

This Indian Standard (Part 3) (Second Revision), which is identical with ISO 6579 : 1993 'Microbiology — General guidance on methods for the detection of *Salmonella*' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Food Microbiology Sectional Committee (FAD 46) and approval of the Food and Agriculture Division Council.

In the adopted standard certain terminology and conventions are not identical with those used in the Indian Standards. Attention is especially drawn to the following:

- a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'.
- b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

This standard was originally published in 1970 and first in 1976 splitting into parts; Part 3 covering the isolation and identification of *Salmonella* and *Shigella*. On a review by the technical committee responsible for formulating standards in the area of food microbiology, the Part 3 is being revised to cover the provisions of *Salmonella* only and to align with ISO 6579 : 1993. The provisions of *Shigella* are covered separately in Part 7.

## CROSS REFERENCES

<i>International Standard</i>	<i>Corresponding Indian Standard</i>	<i>Degree of Equivalence</i>
ISO 6887 : 1983 Microbiology — General guidance for the preparation of dilutions for microbiological examination	IS 10232 : 1982 Guidelines for preparation of dilution for microbiological examination for food	Equivalent
ISO 7218 : 1996 Microbiology of food and animal feeding stuffs — General rules for microbiological examination	IS 5404 : 1984 Code of practice for handling of samples for microbiological analysis ( <i>first revision</i> )	Related

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'.

At present, the Central Research Institute, Kasauli, Microbiology Division of Indian Veterinary Research Institute, Izatnagar serve as National *Salmonella* Centre.

# Indian Standard

## METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

### PART 3 GENERAL GUIDANCE ON METHODS FOR THE DETECTION OF *SALMONELLA* ( Second Revision )

**WARNING** — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials.

#### 1 Scope

This International Standard gives general guidance on methods for the detection of *Salmonella*.

Subject to the limitations discussed in the Introduction, this International Standard is applicable to products intended for human consumption or feeding of animals.

The incubation temperature (35 °C or 37 °C) shall be agreed by the parties concerned and shall be specified in the test report.

#### 2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations*.

#### 3 Definitions

For the purposes of this International Standard, the following definitions apply.

**3.1 *Salmonella*:** Microorganisms which form typical colonies on solid selective media and which display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard.

**3.2 detection of *Salmonella*:** Determination of the presence or absence of these microorganisms, in a particular mass of product, when tests are carried out in accordance with this International Standard.

#### 4 Principle

The detection of *Salmonella* necessitates four successive stages (see also annex A).

NOTE 1 *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other members of *Enterobacteriaceae* or of other families. Therefore, selective enrichment is necessary; furthermore, pre-enrichment is often necessary to permit detection of injured *Salmonella*.

##### 4.1 Pre-enrichment in non-selective liquid medium

Inoculation of buffered peptone water (also used as diluent) with the test portion, and incubation at 35 °C or 37 °C (as agreed) for 16 h to 20 h.

##### 4.2 Enrichment in selective liquid media

Inoculation of magnesium chloride/malachite green medium and of a selenite/cystine medium with the culture obtained in 4.1.

Incubation of the magnesium chloride/malachite green medium at 42 °C for 24 h and incubation of the

selenite/cystine medium at 35 °C or 37 °C (as agreed) for 24 h and a further 24 h.

### 4.3 Plating out and recognition

From the cultures obtained in 4.2, inoculation of two selective solid media:

- phenol red/brilliant green agar, unless the International Standard appropriate to the product to be examined, or other specific considerations (for example the isolation of lactose-positive *Salmonella*), require substitution of some other medium as the one for obligatory use;
- any other solid selective medium (see 5.2.4.2).

Incubation at 35 °C or 37 °C (as agreed), and examination after 24 h and, if necessary, after 48 h to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

### 4.4 Confirmation

Subculturing of colonies of presumptive *Salmonella*, plated out as described in 4.3, and confirmation by means of appropriate biochemical and serological tests.

## 5 Culture media, reagents and sera

### 5.1 General

For current laboratory practice, see ISO 7218.

### 5.2 Culture media and reagents

NOTE 2 Because of the large number of culture media and reagents, it has been considered preferable, for the clarity of the text, to give their composition and preparation in annex B.

#### 5.2.1 Non-selective pre-enrichment medium: Buffered peptone water

See clause B.1.

#### 5.2.2 First selective enrichment medium: Rappaport-Vassiliadis magnesium chloride/malachite green medium (RV medium)

See clause B.2.

#### 5.2.3 Second selective enrichment medium: Selenite/cystine medium

See clause B.3.

### 5.2.4 Solid selective plating-out media

#### 5.2.4.1 First medium: Phenol red/brilliant green agar (Edel and Kampelmacher)

See clause B.4.

This first medium is compulsory unless otherwise stated (see 4.3).

#### 5.2.4.2 Second medium

The choice of the second medium is left to the discretion of the testing laboratory, unless there is a specific International Standard relating to the product to be examined, which specifies the composition of this second medium.

### 5.2.5 Nutrient agar

See clause B.5.

### 5.2.6 Triple sugar/iron agar (TSI agar)

See clause B.6.

### 5.2.7 Urea agar (Christensen)

See clause B.7.

### 5.2.8 L-Lysine decarboxylation medium

See clause B.8.

### 5.2.9 Reagent for detection of $\beta$ -galactosidase (or prepared paper discs, used in accordance with the manufacturer's instructions)

See clause B.9.

### 5.2.10 Reagents for Voges-Proskauer (VP reaction)

See clause B.10.

#### 5.2.10.1 VP medium

#### 5.2.10.2 Creatine solution (*N*-amidinosarcosine)

#### 5.2.10.3 1-Naphthol, ethanolic solution

#### 5.2.10.4 Potassium hydroxide solution

### 5.2.11 Reagents for indole reaction

See clause B.11.

#### 5.2.11.1 Tryptone-tryptophan medium

#### 5.2.11.2 Kovacs reagent (*N,N*-dicyclohexyl-carbodiimide pentachlorophenol complex)

#### 5.2.12 Semi-solid nutrient agar

See clause B.12.

#### 5.2.13 Saline solution

See clause B.13.

### 5.3 Sera

Several types of agglutinant sera containing antibodies for one or several O-antigens are available commercially, i.e. anti-sera containing one or more "O" groups (called monovalent or polyvalent anti-O sera), anti-Vi sera, and anti-sera containing antibodies for one or several H-factors (called monovalent or polyvalent anti-H sera).

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes. Assistance towards this objective may be obtained by using anti-sera prepared by a supplier recognized as competent (for example, by an appropriate government agency).

## 6 Apparatus and glassware

NOTE 3 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment and, in particular, the following.

#### 6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave)

See ISO 7218.

#### 6.2 Drying cabinet or oven, ventilated by convection, capable of operating between $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and $55\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ .

#### 6.3 Incubator, capable of operating at $35\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ or $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , depending on the temperature agreed.

#### 6.4 Water bath, capable of operating at $42,0\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ or incubator, capable of operating at $42,0\text{ }^{\circ}\text{C} \pm 0,5\text{ }^{\circ}\text{C}$ .

#### 6.5 Water baths, capable of operating at $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , $55\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and $70\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ .

#### 6.6 Water bath, capable of operating at $35\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ or $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , depending on the temperature agreed.

#### 6.7 Loops, made of platinum/iridium or nickel/ chromium, of diameter approximately 3 mm.

#### 6.8 pH-meter, having an accuracy of calibration of $\pm 0,1$ pH unit at $25\text{ }^{\circ}\text{C}$ .

#### 6.9 Culture bottles or flasks

NOTE 4 Bottles or flasks with non-toxic metallic or plastic screw-caps may be used.

#### 6.10 Culture tubes, 8 mm in diameter and 160 mm in length.

#### 6.11 Measuring cylinders

#### 6.12 Graduated pipettes, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

#### 6.13 Petri dishes, of small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

## 7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage

Sampling is not part of the method specified in this International Standard. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

## 8 Preparation of the test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

## 9 Procedure

(See diagram in annex A.)

### 9.1 Test portion and initial suspension

#### 9.1.1 See ISO 6887 and the specific International Standard dealing with the product concerned.

For preparation of the initial suspension, use as dilution fluid the pre-enrichment medium specified in 5.2.1.

**9.1.2** In general, to prepare the initial suspension, add a 25 g test portion to 225 ml of pre-enrichment medium (5.2.1), which is the ratio of test portion to pre-enrichment medium specified in this method.

If the prescribed test portion is other than 25 g, use the necessary quantity of pre-enrichment medium to yield approximately a 1/10 dilution (mass to volume).

#### NOTES

5 To reduce the examination workload when more than one 25 g test portion from a specified lot of food has to be examined, and when evidence is available that compositing (pooling the test portions) does not affect the result for that particular food, the test portions may be composited. For example, if 10 test portions of 25 g are to be examined, combine the 10 units to form a composite test portion of 250 g and add 2,25 litres of pre-enrichment broth. Alternatively, the 0,1 ml (RV medium) and 10 ml (selenite/cystine medium) portions of the pre-enrichment broths from the 10 separate test portions (9.3.1) may be composited for enrichment in 0,1 litre and 1 litre respectively of selective enrichment medium.

6 Dried or powdered food products may need a special rehydration procedure to enhance the recovery of *Salmonella*. Two techniques may be used for this purpose, that of immersion and that of agitation. Refer for this purpose to the specific International Standard dealing with the product under examination. If such a standard is not available, it is recommended that the parties concerned come to an agreement on this subject.

## 9.2 Non-selective pre-enrichment

Incubate the initial suspension at 35 °C or 37 °C (as agreed) for not less than 16 h and not more than 20 h.

## 9.3 Selective enrichment

**9.3.1** Transfer 0,1 ml of the culture obtained in 9.2 to a tube containing 10 ml of the RV medium (5.2.2); transfer 10 ml of the culture obtained in 9.2 to a flask containing 100 ml of selenite/cystine medium (5.2.3).

**9.3.2** Incubate the two inoculated media (9.3.1) for 18 h to 24 h as follows:

- a) the inoculated RV medium at 42 °C for 24 h;
- b) the inoculated selenite/cystine medium at 35 °C or 37 °C (as agreed) for 24 h and a further 24 h.

**NOTE 7** For the selenite/cystine medium, it may, in some cases, be advantageous to raise the incubation temperature to 42 °C. This modification should be indicated in the test report.

## 9.4 Plating out and identification

**9.4.1** Using the culture obtained in the RV medium, after incubation for 24 h, inoculate, by means of a loop (6.7), the surface of one large-size Petri dish (6.13) containing the first selective plating-out medium (generally the phenol red/brilliant green agar, see 5.2.4.1), so that well-isolated colonies will be obtained.

In the absence of large dishes, use two small dishes, one after the other, using the same loop (see note 8).

Proceed in the same way with the second selective plating-out medium (5.2.4.2) using a new loop and Petri dishes of appropriate size.

**NOTE 8** The following method of streaking is recommended when phenol red/brilliant green agar is used. Use one loop (6.7) for two dishes. Take a droplet from the edge of the surface of the fluid. Inoculate both dishes according to the two diagrams in annex D. Use the whole dish; loop streaks should be spaced about 0,5 cm apart. (Do not flame the loop or recharge it after making the first streak, nor when passing to the second dish.) When only one large dish is used, the method of streaking should be as indicated for the first dish in annex D.

**9.4.2** Using the culture obtained in the selenite/cystine medium after incubation for 24 h, repeat the procedure described in 9.4.1 with the two selective plating-out media.

**9.4.3** Invert the dishes (9.4.1) and (9.4.2) so that the bottom is uppermost, and place them in the incubator (6.3) set at 35 °C or 37 °C (as agreed).

**9.4.4** After a total incubation period of 48 h of the selenite/cystine medium (see 9.3.2 and 9.4.3), repeat the procedure described in 9.4.2 and 9.4.3.

**9.4.5** After incubation for 20 h to 24 h, examine the dishes (9.4.3 and 9.4.4) for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on phenol red/brilliant green agar cause the colour of the medium to change from pink to red.

**9.4.6** If growth is slight or if no typical colonies of *Salmonella* are present, reincubate at 35 °C or at 37 °C (as agreed) for a further 18 h to 24 h.

Re-examine the plates for the presence of typical colonies of *Salmonella*.

**NOTE 9** Any typical or suspect colony should be subjected to a confirmation (9.5); the recognition of colonies of *Salmonella* is to a large extent a matter of experience, and their appearance may vary somewhat, not only from species to species, but also from batch to batch of medium. In this respect, agglutination, at this stage, of colonies with polyvalent *Salmonella* anti-serum may facilitate recognition of suspected colonies.

**9.4.7** Identification kits currently available commercially and permitting the identification of *Salmonella* may be used.

## 9.5 Confirmation

### 9.5.1 Selection of colonies for confirmation

For confirmation, take from each dish of each selective medium (see 9.4.5 and 9.4.6), five colonies considered to be typical or suspect.

If on one dish there are fewer than five typical or suspect colonies, take for confirmation all the typical or suspect colonies.

Streak the selected colonies onto the surface of pre-dried nutrient agar plates (5.2.5), in a manner which will allow well-isolated colonies to develop.

Incubate the inoculated plates at 35 °C or 37 °C (as agreed) for 18 h to 24 h.

Use pure cultures for biochemical and serological confirmation.

### 9.5.2 Biochemical confirmation

By means of an inoculating wire, inoculate the media specified in 9.5.2.1 to 9.5.2.6 with each of the cultures obtained from the colonies selected in 9.5.1.

#### 9.5.2.1 TSI agar (5.2.6)

Streak the agar slope surface and stab the butt.

Incubate at 35 °C or 37 °C (as agreed) for 24 h.

Interpret the changes in the medium as follows:

#### Butt

yellow:	glucose positive (fermentation of glucose)
red or unchanged.	glucose negative (no fermentation of glucose)
black:	formation of hydrogen sulphide
bubbles or cracks:	gas formation from glucose

#### Slant surface

yellow:	lactose and/or sucrose positive (lactose and/or sucrose used)
red or unchanged:	lactose and sucrose negative (neither lactose nor sucrose used)

Typical *Salmonella* cultures show alkaline (red) slants with gas formation and acid (yellow) butts, with (in about 90 % of the cases) formation of hydrogen sulphide (blackening of the agar).

When a lactose-positive *Salmonella* is isolated (see 4.3), the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar test only (see 9.5.3).

#### 9.5.2.2 Urea agar (5.2.7)

Streak the agar slope surface.

Incubate at 35 °C or 37 °C (as agreed) for 24 h and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h.

#### 9.5.2.3 L-Lysine decarboxylation medium (5.2.8)

Inoculate just below the surface of the liquid medium.

Incubate at 35 °C or 37 °C (as agreed) for 24 h.

A purple colour after incubation indicates a positive reaction.

A yellow colour indicates a negative reaction.

#### 9.5.2.4 Detection of $\beta$ -galactosidase (5.2.9)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (5.2.13).

Add 1 drop of toluene and shake the tube.

Put the tube in a water bath (6.6) set at 35 °C or 37 °C (as agreed) and leave for several minutes.

Add 0,25 ml of the reagent for detection of  $\beta$ -galactosidase and mix.

Replace the tube in the water bath set at 35 °C or 37 °C (as agreed), leave for 24 h, examining the tube at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

If prepared paper discs (5.2.9) are used, follow the manufacturer's instructions.

#### 9.5.2.5 Medium for Voges-Proskauer (VP) reaction (5.2.10)

Suspend a loopful of the suspected colony in a sterile tube containing 0,2 ml of the VP medium (5.2.10.1).

Incubate at 35 °C or 37 °C (as agreed) for 24 h.

After incubation, add two drops of the creatine solution (5.2.10.2), three drops of the ethanolic solution of 1-naphthol (5.2.10.3) and then two drops of the potassium hydroxide solution (5.2.10.4); shake after the addition of each reagent.

The formation of a pink to bright red colour within 15 min indicates a positive reaction.

#### 9.5.2.6 Medium for indole reaction (5.2.11)

Inoculate a tube containing 5 ml of the tryptone/tryptophan medium (5.2.11.1) with the suspected colony.

Incubate at 35 °C or 37 °C (as agreed) for 24 h.

After incubation, add 1 ml of the Kovacs reagent (5.2.11.2).

The formation of a red ring indicates a positive reaction.

A yellow-brown ring indicates a negative reaction.

#### 9.5.2.7 Interpretation of the biochemical tests

*Salmonella* generally show the reactions given in table 1.

#### 9.5.3 Serological confirmation

The detection of the presence of *Salmonella* O-, Vi- and H-antigens is tested by slide agglutination with the appropriate sera, from pure colonies (9.5.1) and after auto-agglutinable strains have been eliminated.

##### 9.5.3.1 Elimination of auto-agglutinable strains

Place one drop of the saline solution (5.2.13) onto a carefully cleaned glass slide.

Disperse in this drop part of the colony to be tested, so as to obtain a homogeneous and turbid suspension.

Rock the slide gently for 30 s to 60 s.

Observe the result against a dark background, preferably with the aid of a magnifying glass.

If the bacteria have clumped into more or less distinct units, the strain is considered auto-agglutinable, and shall not be submitted to the following tests as the detection of the antigens is impossible.

Table 1

Test <sup>1)</sup>	Positive or negative reaction	Percentage of <i>Salmonella</i> inoculations showing the reaction <sup>2)</sup>
TSI glucose (acid formation) (9.5.2.1)	+	100
TSI glucose (gas formation) (9.5.2.1)	+	91,9 <sup>3)</sup>
TSI lactose (9.5.2.1)	—	99,2 <sup>4)</sup>
TSI sucrose (9.5.2.1)	—	99,5
TSI hydrogen sulfide (9.5.2.1)	+	91,6
Urea splitting (9.5.2.2)	—	99
Lysine decarboxylation (9.5.2.3)	+	94,6 <sup>5)</sup>
$\beta$ -Galactosidase reaction (9.5.2.4)	—	98,5 <sup>4)</sup>
Voges-Proskauer reaction (9.5.2.5)	—	100
Indole reaction (9.5.2.6)	—	98,9

1) Ewing W. H. and Ball M. M., *The biochemical reactions of members of the genus Salmonella*. National Communicable Disease Center, Atlanta, Georgia, USA (1966).

2) These percentages indicate only that not all strains of *Salmonella* show the reactions marked + or —. These percentages may vary from country to country and from food product to food product.

3) *Salmonella typhi* is anaerogenic.

4) The *Salmonella* subgenus III (Arizona) gives positive or negative lactose reactions but is always  $\beta$ -galactosidase-positive. The *Salmonella* subgenus II gives a negative lactose reaction, but gives a positive  $\beta$ -galactosidase reaction. For the study of strains, it may be useful to carry out complementary biochemical tests.

5) *S. paratyphi* A is negative.

##### 9.5.3.2 Examination for O-antigens

Using one pure colony recognized as non-auto-agglutinable, proceed according to 9.5.3.1, using one drop of the anti-O serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

Use the poly- and monovalent sera one after the other.

##### 9.5.3.3 Examination for Vi-antigens

Proceed according to 9.5.3.1, but using one drop of the anti-Vi serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

#### 9.5.3.4 Examination for H-antigens

Inoculate the semi-solid nutrient agar (5.2.12) with a pure non-auto-agglutinable colony.

Incubate the medium at 35 °C or 37 °C (as agreed) for 18 h to 24 h.

Use this culture for examination for the H-antigens, proceeding according to 9.5.3.1, but using one drop of the anti-H serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

#### 9.5.4 Interpretation of biochemical and serological reactions

Table 2 gives the interpretation of the confirmatory tests (9.5.2 and 9.5.3) carried out on the colonies used (9.5.1).

Table 2

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O-, Vi- or H-antigen positive	Strains considered to be <i>Salmonella</i>
Typical	No	All reactions negative	May be <i>Salmonella</i>
Typical	Yes	Not tested (see 9.5.3.1)	
No typical reactions	No	O-, Vi- or H-antigen positive	
No typical reactions	No	All reactions negative	Not considered to be <i>Salmonella</i>

#### 9.5.5 Definitive confirmation

Strains which are considered to be *Salmonella*, or which may be *Salmonella* (see table 2), shall be sent

to a recognized *Salmonella* reference centre for definitive typing.

This dispatch shall be accompanied by all possible information concerning the strain(s).

### 10 Expression of results

In accordance with the results of the interpretation, indicate the presence or absence of *Salmonella* in a test portion of x g of product.

### 11 Test report

The test report shall specify the method used and the results obtained. It shall also mention all operating conditions not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

It shall specify, in particular, the incubation temperature used, i.e. 35 °C or 37 °C, and, in the case of the selenite/cystine medium, whether the temperature was raised to 42 °C.

The test report shall also state whether a positive result was obtained only when using a plating-out medium (5.2.4) not specified in this International Standard.

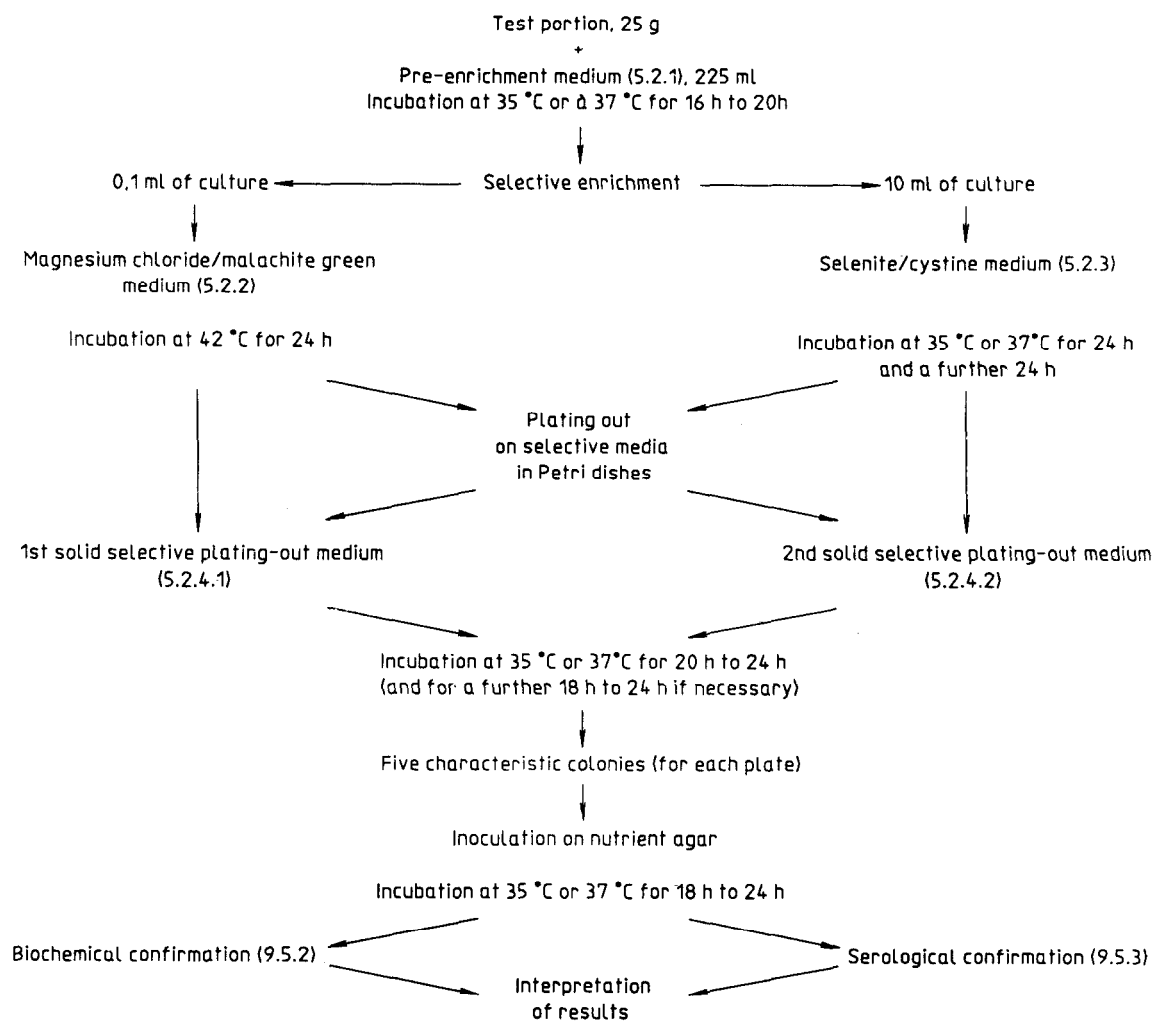
The test report shall include all information necessary for the complete identification of the sample.

### 12 Quality assurance

To check the ability of the laboratory to detect *Salmonella* with the methods and media described in this International Standard, introduce reference samples into control flasks of the pre-enrichment medium (see 5.2.1). Proceed with the control flasks as for the test cultures.

## Annex A (normative)

### Diagram of procedure



## Annex B (normative)

### Composition and preparation of culture media and reagents

#### B.1 Buffered peptone water

##### B.1.1 Composition

Peptone	10,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate dodecahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ )	9,0 g
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	1,5 g
Water	1 000 ml

##### B.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0.

Dispense the medium into flasks of suitable capacity to obtain the portions necessary for the test.

Sterilize in the autoclave (6.1) set at 121 °C for 20 min.

#### B.2 Rappaport-Vassiliadis magnesium chloride-malachite green medium

##### B.2.1 Solution A

###### B.2.1.1 Composition

Tryptone	5,0 g
Sodium chloride	8,0 g
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	1,6 g
Water	1 000 ml

###### B.2.1.2 Preparation

Dissolve the components in the water by heating to about 70 °C.

The solution shall be prepared on the day of preparation of the RV medium.

##### B.2.2 Solution B

###### B.2.2.1 Composition

Magnesium chloride hexahydrate ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ )	400,0 g
Water	1 000 ml

###### B.2.2.2 Preparation

Dissolve the magnesium chloride in the water.

As this salt is very hygroscopic, it is advisable to dissolve the entire contents of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  from a newly opened container, according to the formula. For instance, 250 g of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  is added to 625 ml of water, giving a solution of total volume of 795 ml and a concentration of about 31,5 g percent of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ .

The solution can be kept in a brown glass bottle at room temperature.

##### B.2.3 Solution C

###### B.2.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

###### B.2.3.2 Preparation

Dissolve the malachite green oxalate in the water.

The solution can be kept in a brown glass bottle at room temperature.

##### B.2.4 Complete medium

###### B.2.4.1 Composition

Solution A (B.2.1)	1 000 ml
Solution B (B.2.2)	100 ml
Solution C (B.2.3)	10 ml

#### B.2.4.2 Preparation

Add to 1 000 of solution A, 100 ml of solution B and 10 ml of solution C.

Adjust the pH, if necessary, so that after sterilization it is 5,2.

Distribute before use into test tubes in 10 ml quantities.

Sterilize in the autoclave (6.1) set at 115 °C for 15 min.

Store the prepared medium in the refrigerator.

### B.3 Selenite/cystine medium

#### B.3.1 Base

##### B.3.1.1 Composition

Tryptone	5,0 g
Lactose	4,0 g
Disodium hydrogen phosphate dodecahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ )	10,0 g
Sodium hydrogen selenite	4,0 g
Water	1 000 ml

##### B.3.1.2 Preparation

Dissolve the first three basic components in the water by boiling for 5 min. After cooling, add the sodium hydrogen selenite.

Adjust the pH, if necessary, so that it is 7,0.

#### B.3.2 L-Cystine solution

##### B.3.2.1 Composition

L-Cystine	0,1 g
Sodium hydroxide solution, $c(\text{NaOH}) = 1 \text{ mol/l}$	15 ml
Sterile water, to a final volume of	100 ml

##### B.3.2.2 Preparation

Place the components in a sterile volumetric flask.

Dilute to 100 ml with sterile water.

Do not sterilize.

### B.3.3 Complete medium

#### B.3.3.1 Composition

Base (B.3.1)	1 000 ml
L-Cystine solution (B.3.2)	10 ml

##### B.3.3.2 Preparation

Cool the base and add the L-cystine solution aseptically.

Adjust the pH, if necessary, so that it is 7,0.

Dispense the medium aseptically into sterile flasks of suitable capacity to obtain the portions necessary for the test.

Use the medium on the day of preparation.

### B.4 Phenol red/brilliant green agar (Edel and Kampelmacher)

#### B.4.1 Base

##### B.4.1.1 Composition

Meat extract powder	5,0 g
Peptone	10,0 g
Yeast extract powder	3,0 g
Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ )	1,0 g
Sodium dihydrogen phosphate ( $\text{NaH}_2\text{HPO}_4$ )	0,6 g
Agar in powder or in flake form	12 g to 18 g <sup>1)</sup>
Water	900 ml

1) Depending on the gel strength of the agar.

##### B.4.1.2 Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0.

Transfer the base to tubes or flasks of appropriate capacity.

Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

## B.4.2 Sugar/phenol red solution

### B.4.2.1 Composition

Lactose	10,0 g
Sucrose	10,0 g
Phenol red	0,09 g
Water, to a final volume of	100 ml

### B.4.2.2 Preparation

Dissolve the components in about 50 ml the water in a volumetric flask.

Make up to 100 ml with the water.

Heat in a water bath at 70 °C for 20 min.

Cool to 55 °C ± 1 °C and use immediately.

## B.4.3 Brilliant green solution

### B.4.3.1 Composition

Brilliant green (see specifications in annex C)	about 0,5 g
Water	100 ml

### B.4.3.2 Preparation

Add the brilliant green to the water.

Store the solution for at least 1 day in the dark to allow auto-sterilization to occur.

## B.4.4 Complete medium

### B.4.4.1 Composition

Base (B.4.1)	900 ml
Sugar/phenol red solution (B.4.2)	100 ml
Brilliant green solution (B.4.3)	1 ml

### B.4.4.2 Preparation

Add, under aseptic conditions, the brilliant green solution to the sugar/phenol red solution cooled to 55 °C ± 1 °C.

Add it all to the base at 50 °C to 55 °C and mix.

## B.4.5 Preparation of the agar plates

Place in each of an appropriate number of large Petri dishes (6.13) about 40 ml of the freshly prepared complete medium (B.4.4). (If large dishes are not available, place about 15 ml of the medium in small Petri dishes.) Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.2) set between 37 °C and 55 °C until the surface of the agar is dry.

If prepared in advance, the undried agar plates shall be kept for no longer than 4 h at room temperature or one day in the refrigerator.

## B.5 Nutrient agar

### B.5.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	12 g to 18 g <sup>1)</sup>
Water	1 000 ml

1) Depending on the gel strength of the agar.

### B.5.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0.

Transfer the culture medium into tubes or bottles of appropriate capacity.

Sterilize in the autoclave (6.1) set at 121 °C for 20 min.

### B.5.3 Preparation of nutrient agar plates

Transfer about 15 ml of the melted medium to sterile small Petri dishes (6.13) and proceed as in B.4.5.

## B.6 Triple sugar/iron agar (TSI agar)

### B.6.1 Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Iron(III) citrate	0,3 g
Sodium thiosulfate	0,3 g
Phenol red	0,024 g
Agar	12 g to 18 g <sup>1)</sup>
Water	1 000 ml

1) Depending on the gel strength of the agar.

### B.6.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,4.

Dispense the medium in quantities of 10 ml into test tubes.

Sterilize in the autoclave (6.1) set at 121 °C for 10 min.

Allow to set in a sloping position to give a butt of depth 2,5 cm.

### B.7.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8.

Sterilize in the autoclave (6.1) set at 121 °C for 20 min.

### B.7.2 Urea solution

#### B.7.2.1 Composition

Urea	400 g
Water, to a final volume of	1 000 ml

#### B.7.2.2 Preparation

Dissolve the urea in the water.

Sterilize by filtration and check the sterility.

(For details of the technique of sterilization by filtration, refer to any appropriate textbook on microbiology.)

### B.7.3 Complete medium

#### B.7.3.1 Composition

Base (B.7.1)	950 ml
Urea solution (B.7.2)	50 ml

#### B.7.3.2 Preparation

Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to 45 °C ± 1 °C.

Dispense the complete medium in quantities of 10 ml into sterile tubes.

Allow to set in a sloping position.

## B.8 L-Lysine decarboxylation medium

### B.8.1 Composition

L-Lysine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Water	1 000 ml

## B.8.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8.

Transfer the medium in quantities of 5 ml to narrow culture tubes (6.10).

Sterilize in the autoclave (6.1) set at 121 °C for 10 min.

## B.9 $\beta$ -Galactosidase reagent

### B.9.1 Buffer solution

#### B.9.1.1 Composition

Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ )	6,9 g
Sodium hydroxide, 10 mol/l solution	about 3 ml
Water, to a final volume of	50 ml

#### B.9.1.2 Preparation

Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a volumetric flask.

Adjust the pH to 7,0 with the sodium hydroxide solution.

Add water to a final volume of 50 ml.

### B.9.2 ONPG solution

#### B.9.2.1 Composition

<i>o</i> -Nitrophenyl $\beta$ -D-galactopyranoside (ONPG)	0,08 g
Water	15 ml

#### B.9.2.2 Preparation

Dissolve the ONPG in the water at 50 °C  $\pm$  1 °C.

Cool the solution.

### B.9.3 Complete reagent

#### B.9.3.1 Composition

Buffer solution (B.9.1)	5 ml
ONPG solution (B.9.2)	15 ml

## B.9.3.2 Preparation

Add the buffer solution to the ONPG solution.

## B.10 Reagents for Voges-Proskauer (VP) reaction

### B.10.1 VP medium

#### B.10.1.1 Composition

Peptone	7,0 g
Glucose	5,0 g
Dipotassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4$ )	5,0 g
Water	1 000 ml

#### B.10.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,9.

Transfer 3 ml of the medium into each of several tubes.

Sterilize in the autoclave (6.1) set at 115 °C for 20 min.

### B.10.2 Creatine solution (*N*-amidinosarcosine)

#### B.10.2.1 Composition

Creatine monohydrate	0,5 g
Water	100 ml

#### B.10.2.2 Preparation

Dissolve the creatine monohydrate in the water.

### B.10.3 1-Naphthol, ethanolic solution

#### B.10.3.1 Composition

1-Naphthol	6 g
Ethanol, 96 % (V/V)	100 ml

#### B.10.3.2 Preparation

Dissolve the 1-naphthol in the ethanol.

## B.10.4 Potassium hydroxide solution

### B.10.4.1 Composition

Potassium hydroxide	40 g
Water	100 ml

### B.10.4.2 Preparation

Dissolve the potassium hydroxide in the water.

## B.11 Reagents for indole reaction

### B.11.1 Tryptone/tryptophan medium

#### B.11.1.1 Composition

Tryptone	10 g
Sodium chloride	5 g
DL-Tryptophan	1 g
Water	1 000 ml

#### B.11.1.2 Preparation

Dissolve the components in the water at 100 °C.

Adjust the pH, if necessary, so that after sterilization it is 7,5.

Dispense 5 ml of the medium into each of several tubes.

Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

### B.11.2 Kovacs reagent

#### B.11.2.1 Composition

4-Dimethylaminobenzaldehyde	5 g
Hydrochloric acid, $\rho = 1,18 \text{ g/ml}$ to $1,19 \text{ g/ml}$	25 ml
2-Methylbutan-2-ol	75 ml

#### B.11.2.2 Preparation

Mix the components.

## B.12 Semi-solid nutrient agar

### B.12.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	4 g to 9 g <sup>1)</sup>
Water	1 000 ml

1) Depending on the gel strength of the agar.

### B.12.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0.

Transfer the medium to flasks of appropriate capacity.

Sterilize at 121 °C for 20 min.

### B.12.3 Preparation of agar plates

Place in small sterile Petri dishes (6.13) about 15 ml of the freshly prepared medium. The agar plates shall not be dried.

## B.13 Saline solution

### B.13.1 Composition

Sodium chloride	8,5 g
Water	1 000 ml

### B.13.2 Preparation

Dissolve the sodium chloride in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0.

Dispense quantities of the solution into flasks or tubes so that they will contain 90 ml to 100 ml after sterilization.

Sterilize in the autoclave (6.1) set at 121 °C for 20 min.

## **Annex C**

(normative)

### **Specification for brilliant green**

#### **C.1 Bacteriological performance**

Suppression of spreading of *Proteus* on phenol red/brilliant green agar (5.2.4.1) while the growth of *Salmonella* is not inhibited.

#### **C.2 Method of test**

##### **C.2.1 Medium**

Prepare the phenol red/brilliant green agar plates according to clause B.4, but with various concentrations of brilliant green within the range 4,5 mg/l to 6 mg/l.

##### **C.2.2 Procedure**

Inoculate one set of agar plates having different concentrations of brilliant green with a pure culture of a swarming *Proteus*, and another similar set with a pure culture of *Salmonella*, and incubate these plates at 35 °C or 37 °C (as agreed) for no longer than 24 h.

A satisfactory concentration of brilliant green should allow growth of *Salmonella* with typical pink colonies, 1 mm to 2 mm in diameter, and limited growth of *Proteus*; i.e. no spreading.

The concentration of brilliant green which shows this pattern should be used for the preparation of the brilliant green solution (see B.4.3).

## **Annex D** (informative)

### **Standard method of streaking agar plates**

